122. The method of Claim 54, wherein said amino acid changes are introduced at random.

123. The method of Claim 122, wherein said amino acid changes are produced by mutagenic PCR, DNA shuffling, or phage display methodology.

124. The method of Claim 123, wherein said engineered protein is confirmed as having the conformation of said native protein by filter lift assay or ELISA.

REMARKS

Claims 54-118 are pending in the application. Claims 59, 66, 73, 74, 81, 84-96, 106, 107, and 108-118 have been cancelled. New claims 119-124 have been added. Support for the newly added claims can be found in the original claims as well as in the specification. No new matter has been added by way of amendment. Reexamination and reconsideration of the claims are respectfully requested.

Applicants herewith submit a complete set of formal drawings to be considered by the Draftsman.

The detailed action states (page 3, #5) that the application fails to fully comply with the applicable requirements because sequences were disclosed in the application without benefit of SEQ ID NOs. The specification has been amended to include identification of the sequences by SEQ ID NO so as to correlate the sequence listing with the description in the specification. No new matter has been added by way of amendment. Accordingly, this defect of the application has been corrected.

The Objections to the Specification Should Be Withdrawn

The Office Action (11/19/02, page 4, #6) objects to the drawings for having inappropriate labels. Figures 1 and 1A have been relabeled as Figures 1A and 1B, and the brief description of the drawings on page 3 of the specification has been corrected.

The Office Action (page 4, #7) objects to the specification for being confusing with respect to the sequence listing. The specification has been amended to include identification of the sequences by SEQ ID NO. No new matter has been added by way of amendment.

The Office Action (page 4, #8) objects to the Abstract for not completely describing the disclosed subject matter. The Abstract has been amended in accordance with the suggestions in the Office Action.

The Office Action (page 4, #9) objects to the specification due to various specified typographical errors, which have been corrected.

For the above reasons, Applicants believe that the objections to the specification have been obviated by amendment and should be withdrawn.

The Objections to the Claims Should Be Withdrawn

The Office Action (11/19/02, page 5, #10) objects to various claims due to various specified typographical errors, which have been corrected.

The Office Action (page 5, #11) objects to claims 59, 74, and 99 as being of improper dependent form for failing to further limit the subject matter of a previous claim. Claims 59 and 74 have been cancelled, while claim 99 has been amended to recite different limitations.

The Office Action (page 6, #12) objects to claims 70, 115, and 116 as being substantial duplicates of claims 58, 69, and 71, respectively. Claims 115 and 116 have been cancelled. Claims 58 and 69 (and therefore claims 70 and 71, which remain dependent on claim 69) have all been amended to include additional limitations as further described herein. Applicants believe that these changes should obviate the objection to these claims.

As detailed above, Applicants have corrected various specified errors and made claim amendments that address the objections to the claims. Accordingly, Applicants submit that the objections to the claims should be withdrawn.

The Rejection of Claims Under 35 U.S.C.§112, Second Paragraph, Should Be Withdrawn

The Office Action (11/19/02, page 6, #13) has rejected claims 54-68, 117, and 118 as being indefinite due to their recitation of the phrase "whose conformation is unavailable." Claims 117 and 118 have been cancelled. Independent claims 54 and 58 (and thereby claims 55-57, which are dependent on or incorporate the limitations of claim 54 and claims 59-68, which are dependent on or incorporate the limitations of claim 58) have been amended to delete this phrase. Accordingly, this rejection should be withdrawn and not applied to the newly added claims.

The Office Action (page 7, #14) has rejected claims 57 and 72 as indefinite due to their recitation of the term "dimerizing proteins." Applicants respectfully disagree with this rejection. However, in order to advance prosecution, claims 57 and 72 have been amended to recite that the interacting molecules are proteins that form homodimers or heterodimers with said native protein of interest wherein said proteins are not antibodies. Similarly, new claim 119 has been added to claim the specific embodiment of the method of claim 57 wherein said native protein of interest is VSPα or VSPβ and said proteins that form homodimers or heterodimers with said native protein of interest are VSPα or VSPβ.

Applicants note that the specification teaches the use of proteins having affinity to the protein of interest to detect conformational changes, as discussed on page 5 of the specification (see definition of "interacting molecule" and "binding partner"). The specification (page 5) discusses that binding partners or interacting molecules can be "antibodies, monoclonal antibodies, antibody fragments, proteins, modified proteins, nucleotide sequences, such as aptomers, chemical compounds (*e.g.*, carbohydrates, etc.), or combinations thereof." The specification discusses that VSPα and VSPβ proteins can form homodimers or heterodimers (see,

e.g., page 13). Finally, the specification provides working examples in which assays for dimerization of VSPα and VSPβ are performed. In the Experimental section, experiments are described on pages 14 and 21 in which mutagenized VSPβ was screened with VSPα in a filter lift assay for retention of the ability to dimerize with VSPα.

Applicants include copies of several publications with this response to illustrate that the notion of oligomerizing and dimerizing proteins is well-known in the art. Applicants include a copy of DeWald *et al.* (1992) *J. Biol. Chem.* 267: 15958-64, which discusses that VSPα and VSPβ form both homodimers and heterodimers (see, *e.g.*, abstract on first page of reference). Further, to demonstrate that the terms "homodimer" and "heterodimer" are readily understood by those of skill in the art, Applicants also include a copy of the definition of "dimer" from *Dorland's Medical Dictionary* (28th edition 1994, W.B. Saunders Company, Philadelphia, PA) at page 470, which is "a compound formed by combination of two identical simpler molecules." Thus, as used in the present specification and claims, the term "dimer" has the common meaning of a combination of two molecules. Applicants also include copies of the dictionary definitions of the prefixes "homo-" ("a combining form meaning the same") and "hetero-" ("a combining form meaning other, different..."), at pages 773 and 761 of Dorland's Medical Dictionary, respectively. Applicants respectfully submit that it is understood by those of skill in the art, as illustrated by the use of these terms in DeWald *et al.* (see, *e.g.*, the abstract on page 15958), that heterodimers are combinations of two molecules which differ and homodimers are combinations of two like molecules.

For the above reasons, Applicants believe that one of ordinary skill in the art would be readily able to discern the scope of the invention. Accordingly, this rejection under 35 U.S.C. §112, second paragraph, should be withdrawn.

The Office Action (page 8, #15) has rejected claims 65, 80, and 105 as indefinite due to recitation of the term "correctly folded variants." In order to clarify the claim language, these claims have been amended to recite that said engineered protein is confirmed as having the conformation of said native protein by the specified methods.

The Office Action (page 8, #16) has rejected claims 66, 67, 81, 82, 106, and 107 as indefinite due to recitation of the term "increased to represent 5% of the total amino acid content." Claims 66, 81, 106, and 107 have been cancelled. Claims 67 and 82 have been amended in accordance with the Examiner's suggestion to recite that said nutritionally essential amino acids are increased to represent at least a specified percentage of the total amino acid content.

The Rejection of Claims Under 35 U.S.C.§112, First Paragraph, Should Be Withdrawn

Applicants acknowledge with appreciation the withdrawal of the previous rejection of claims 54-83, 97-107, and 115-118 under 35 U.S.C. §112, first paragraph, as lacking enablement (Office Action of 11/19/02, page 9, #18).

The Office Action (11/19/02, page 9) has rejected claims 57 and 72 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time the application was filed. Applicants respectfully disagree. However, in order to advance prosecution, claims 57 and 72 have been amended to recite that the interacting molecules are proteins that form homodimers or heterodimers with said native protein of interest. Similarly, new claim 119 has been added to claim the specific embodiment of the method of claim 57 wherein said native protein of interest is VSPα or VSPβ and said proteins that form homodimers or heterodimers with said native protein of interest are VSPα or VSPβ.

As discussed above (in addressing the rejection of claims 57 and 72 under 35 U.S.C. §112, second paragraph), Applicants note that the specification teaches the use of proteins having affinity to the protein of interest to detect conformational changes, as discussed on page 5 of the specification (see definition of "interacting molecule" and "binding partner"). The specification (page 5) discusses that binding partners or interacting molecules can be "antibodies, monoclonal antibodies, antibody fragments, proteins, modified proteins, nucleotide sequences,

such as aptomers, chemical compounds (*e.g.*, carbohydrates, etc.), or combinations thereof." The specification discusses that VSPα and VSPβ proteins can form homodimers or heterodimers (see, *e.g.*, page 13). Finally, the specification provides working examples in which assays for dimerization of VSPα and VSPβ are performed. In the Experimental section, experiment are described on pages 14 and 21 in which mutagenized VSPβ was screened with VSPα in a filter lift assay for retention of the ability to dimerize with VSPα.

Applicants include copies of several publications with this response to illustrate that the notion of oligomerizing and dimerizing proteins is well-known in the art. Applicants include a copy of DeWald *et al.* (1992) *J. Biol. Chem.* 267: 15958-64, which discusses that VSPα and VSPβ form both homodimers and heterodimers (see, *e.g.*, abstract on first page of reference). Further, to demonstrate that the terms "homodimer" and "heterodimer" are readily understood by those of skill in the art, Applicants also include a copy of the definition of "dimer" from *Dorland's Medical Dictionary* (28th edition 1994, W.B. Saunders Company, Philadelphia, PA) at page 470, which is "a compound formed by combination of two identical simpler molecules." Thus, as used in the present specification and claims, the term "dimer" has the common meaning of a combination of two molecules. Applicants also include the dictionary definitions of the prefixes "homo-" ("a combining form meaning the same") and "hetero-" ("a combining form meaning other, different..."), at pages 773 and 761 of Dorland's Medical Dictionary, respectively. Accordingly, Applicants respectfully submit that it is understood by those of skill in the art, as illustrated by the use of these terms in DeWald *et al.*, that heterodimers are combinations of two molecules which differ and homodimers are combinations of two like molecules.

For the above reasons, Applicants believe that one of ordinary skill in the art would be readily able to appreciate that Applicants had possession of the claimed invention at the time the application was filed. Accordingly, this rejection under 35 U.S.C. §112, first paragraph, should be withdrawn.

The Rejection of Claims Under 35 U.S.C. §102(b) Should Be Withdrawn

The Office Action (11/19/02, page 10, #19) has rejected claims 54-57, 61-62, 64-65, and 117-118 under 35 U.S.C. §102(b) as anticipated by Berkner (U.S. Pat. No. 5,288,629) The Office Action states that Berkner teaches a mutant of human factor VII protein whose recombinant expression levels in Baby Hamster Kidney (BHK) cells are measured by an ELISA using an anti-Factor VII monoclonal antibody. This rejection is respectfully traversed. However, in order to advance prosecution, Applicants have made amendments to the claims as set forth above.

Claims 117 and 118 have been cancelled. Independent claims 54 and 58 (and thereby claims 55-57 as well as newly added claims 119-124 which are dependent on claim 54 and claims 61-62 and 64-65 which are dependent on or incorporate the limitations of claim 58) have been amended to incorporate additional limitations; these limitations are not taught or suggested by the Berkner reference. Because the Berkner reference does not describe all of the elements of the claims, the rejection of the claims under 35 U.S.C. §102 should be withdrawn and not applied to the new claims.

The Rejection of Claims Under 35 U.S.C. §103 Should Be Withdrawn

The Office Action (11/19/02, page 11, #20) has rejected claims 54-62, 64-66, 68-77, 79-81, 83, 97-102, 104-106, and 115-118 under 35 U.S.C. §103(a) over Dyer *et al.* (1995) in view of Goldberg (1991). The Office Action (11/19/02, page 11, #21) has also rejected claims 78 and 103 under 35 U.S.C. §103(a) over Dyer *et al.* (1995) in view of Goldberg (1991) and in view of Arnold *et al.* (1997).

The Office Action states that Dyer *et al.* teach: altering the primary structure of phaseolin, a seed storage protein, to enhance the methionine content of the protein to thereby enhance its nutritional value; utilizing specific knowledge of the protein's structure for determination of the most effective insertion sites for methionines so as to cause the least perturbation of the overall protein structure; increasing the methionine content tenfold, from 3 to 33 per 397 amino acids; and assessing the altered protein's structure using thermal and urea

denaturation monitored by circular dichroism. However, the Office Action states that Dyer *et al.* do not teach assessing the altered protein's structure using monoclonal antibody binding assays.

The Office Action states that Goldberg teaches the effectiveness of antibodies in assessing protein conformation, particularly with ELISA assays, and also that monoclonal antibodies are good conformational probes useful in assessing protein folding. The Office Action concludes that it would have been obvious to one of ordinary skill in the art to combine the teachings of Dyer *et al.* and Goldberg to practice methods of altering seed storage proteins wherein antibodies rather than CD spectra are used to assess protein conformation.

Claims 59, 66, 73, 74, 81, 106, and 115-118 have been cancelled. Independent claims that have been amended include claim 54 (from which depends claims 55-57 and newly-added claims 119-124), claim 58 (from which depends claims 60-65 and 68), claim 69 (from which depends claims 70-72, 75-79, 80, and 83), and claim 97 (from which depends claims 98-105). Independent claims 54, 58, 69, and 97 have been amended to recite, among other things, that said amino acid changes alter the amino acid content of said protein by at least 10%.

In contrast, Dyer *et al.* teach much smaller modifications of the methionine content of the bean storage protein phaseolin. As noted in the Office Action (page 12), Dyer *et al.* teach "'increasing by tenfold the methionine content from the original 3 to 33 per 397 amino acid residues of the mature phaseolin polypeptide.'" However, this increase in methionine content amounts to only 8.3% of the total protein. In contrast, the working examples provided in the present specification provide increases in methionine levels to 9.6%, 14.2%, and 17.9% of the total protein.

Applicants further note that Dyer *et al.* actually teaches away from the use of methods that are not based on "[t]hree-dimensional structural information based on X-ray crystallographic analysis." Dyer *et al.* state (page 667, column I): "efforts toward protein engineering of seed storage proteins **have been frustrated primarily by lack of accurate structural information** of the target proteins **and by the inability to characterize structural alterations** brought about by the introduced mutations" (emphasis added). Applicants also note that the Dyer *et al.* study was published in 1995, four years ***after*** the Goldberg reference cited in the Office Action. The

Office Action concludes that the combination of the teachings of Goldberg and Dyer et al. would have been obvious to one of ordinary skill in the art and that one would have been motivated to practice such methods. However, this conclusion is directly contradicted by the insistence of Dyer *et al.* that X-ray crystallographic structural information was essential to progress and that protein engineering had been frustrated in the absence of such information. Thus, Dyer *et al.*, who must be considered those of skill in the art, **taught away** from the use of methods that did not depend on X-ray crystallographic structural information.

Applicants emphasize that in contrast to the teachings of Dyer *et al.*, the present invention provides the benefit that structural information is **not** required to make and use an altered protein. Therefore, because this combination of references does not render the claims obvious, Applicants respectfully request reconsideration and withdrawal of both rejections under 35 U.S.C. §103(a).

CONCLUSION

In view of the above amendments and remarks, Applicants submit that the objections to the specification and claims and the rejections of the claims under 35 U.S.C. §§112, first and second paragraphs, 102(b), and 103(a) are overcome. Applicants respectfully submit that this application is now in condition for allowance. Early notice to this effect is solicited.

If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject Application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those, which may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required

therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner For Patents, Washington, DC 20231, on January 31, 2003.

Lynda-Jo Pixley

RTA/2130560v1

Version with Markings to Show Changes Made:

In the specification:

Please revise the first full paragraph beginning on page 3, line 2, as follows:

[Fig.] Figures 1A and 1B [shows] show [VSP] homologies between vegetative storage protein (VSP) and other proteins, as follows.

Please revise the first full paragraph beginning on page 3, line 3, as follows:

VSP-b (same as VSPβ) and VSP-a (same as VSPα): Staswick, P.E., (1988), Plant Physiol. 87, 250-254. The amino acid sequence of the VSP-b protein is set forth in SEQ ID NO:1, and the amino acid sequence of the VSP-a protein is set forth in SEQ ID NO:2.

Please revise the first full paragraph beginning on page 3, line 6, as follows:

T.phos (tomato acid phosphatase): Erion, J.L., Ballo, B., May, L., Bussell, J., Fox, T.W., & Thomas, S.R., SwissProt database accession number P27061. The amino acid sequence of this protein is set forth in SEQ ID NO:3.

Please revise the first full paragraph beginning on page 3, line 9, as follows:

Ph.vulg (Phaseolus vulgaris): Zhon, P-Y., Tanaka, T., Yamauchi, D., & Minamikawa, T. (1997), Plant Physiol. 113, 479-485. The amino acid sequence of this protein is set forth in SEQ ID NO:4.

Please revise the first full paragraph beginning on page 3, line 12, as follows:

Ar.VSP (Arabidopsis thaliana): Yu, D.Y., Quigley, F., & Mache, R., EMBL database accession number X79490. The amino acid sequence of this protein is set forth in SEQ ID NO:5.

Please revise the first full paragraph beginning on page 3, line 15, as follows:

Ar.1A-1, Ar17A-1 (Arabidopsis thaliana, floral organs): Utsugi, S., Sakamoto, Ogura, Y., Murata, M., & Motoyoshi, F. (1996) Plant Mol. Biol. 32, 759-765. The amino acid sequence of the "Ar.1A-1" protein is set forth in SEQ ID NO:6, and the amino acid sequence of the "Ar17A-1" protein is set forth in SEQ ID NO:7.

Please revise the first full paragraph beginning on page 3, line 18, as follows:

Fig. 2 shows proposed VSPβ methionine-enriched variants. The amino acid sequence of the "VSPβ -Met10" protein is set forth in SEQ ID NO:8, the amino acid sequence of the "VSPβ -Met20" protein is set forth in SEQ ID NO:9, and the amino acid sequence of the "VSPβ -Met30" protein is set forth in SEQ ID NO:10.

Please revise the first full paragraph beginning on page 3, line 23, as follows:

Fig. 4 shows the VSPβ-met10 nucleotide sequence. The VSPβ -met10 nucleotide sequence is also set forth in SEQ ID NO:11.

Please revise the Abstract, beginning on page 27, line 5, as follows:

Methods and compositions for altering amino acid composition of a protein of interest are provided, particularly proteins whose three-dimensional structure is unknown. The method comprises creating interacting molecules to the native protein and selecting for engineered proteins which retain the native conformation by antibody binding. In this manner, the levels of essential amino acids in a protein can be increased yet the biological activity of the protein maintained. Also provided is an exemplary plant protein--*Glycine max* vegetative storage protein (VSP)--in which methionine levels have been increased.

Please revise the first full paragraph beginning on page 8, line 14, as follows:

The transcriptional cassette will include the in [5□-3□] 5′-3′ direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of

interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of A. tumefaciens, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau et al., (1991) Mol. Gen. Genet. 262:141-144; Proudfoot (1991) Cell 64:671-674; Sanfacon et al. (1991) Genes Dev. 5:141-149; Mogen et al. (1990) Plant Cell 2:1261-1272; Munroe et al. (1990) Gene 91:151-158; Ballas et al. 1989) Nucleic Acids Res. 17:7891-7903; Joshi et al. (1987) Nucleic Acid Res. 15:9627-9639.

Please revise the first full paragraph beginning on page 18, line 4, to read as follows:

(a) Conserved residues ([highlighted in blue] shown in Fig. 1) were defined as those residues occurring in more than 5 of the 7 homologs. These were not targeted for substitution. The exceptions were: at residue numbers 19, 37, 146 and 179 (one of the homologs contained a methionine residue); at positions 67, 80, 130 and 169 (conserved hydrophobic amino acid exchanges observed in at least one sequence) and at position 50 (non-conservative changes from Asn to Ser/Cys in two sequences).

Please revise the first full paragraph beginning on page 21, line 6, as follows:

Fifty E. coli colonies containing randomly mutated VSPβ genes were picked as small patches to an SB agar plate containing glucose and ampicillin. Patches were allowed to grow overnight at [37□C] 37°C and were then transferred to a nitrocellulose filter. On the surface of an SB agar plate containing ampicillin and IPTG, this filter was placed on top (cell-side up) of a separate blocked filter to which the antigen (e.g., VSPα) had been coated. During an overnight incubation at [30□C] 30°C, the cells expressed the VSPβ variant they encoded. These proteins were able to diffuse through the top filter and, if correctly folded, bind the antigen-coated filter below. The next day, the antigen-coated filter was washed with PBS-0.05% [tween] Tween™ and incubated with HRP/anti-e tag conjugate. Since the VSPβ mutants are cloned into the pCANTAB-5E vector which fuses a C-terminal epitope tag (e-tag) to the VSPβ protein variants, bound proteins were detected by this antibody in combination with enhanced chemiluminescence detection.

In the claims:

54. (Amended) A method for altering the amino acid composition of a native protein of interest [whose conformation is unavailable], said method comprising introducing amino acid changes into said protein to create an engineered protein, wherein:

a) said amino acid changes alter the amino acid content of said protein by at least 10%;

b) said engineered protein [having] has the conformation of the native protein; and

c) [wherein] said conformation of the engineered protein is confirmed by binding said engineered protein with a set of interacting molecules capable of binding with the native protein, [and] wherein said molecules recognize native conformation.

57. (Amended) The method of Claim 54 wherein said interacting molecules are [dimerizing proteins] proteins that form homodimers or heterodimers with said native protein of interest, wherein said proteins are not antibodies.

58. (Twice amended) A method for altering the amino acid composition of a [native protein of interest whose conformation is unavailable] vegetative storage protein, said method comprising introducing amino acid changes into said protein to create an engineered protein, wherein:

a) said amino acid changes alter the amino acid content of said protein by at least 10%;

b) said engineered protein [having] has the conformation of the native protein;

c) [wherein] said conformation of the engineered protein is confirmed by binding said engineered protein with a [set of antibodies] set of interacting molecules capable of binding with the native protein[,];

d) [wherein] said [antibodies] interacting molecules recognize native conformation and are proteins that form homodimers or heterodimers with said native protein of interest, wherein said interacting molecules are not antibodies [, and wherein said amino acid changes are made to increase levels of nutritionally essential amino acids in the engineered protein].

60. (Amended) The method of Claim 58, wherein said amino acid changes [[involve]]comprise increasing the levels of methionine.

61. (Amended) The method of Claim [54] 58, wherein said amino acid changes are introduced into predetermined sites.

62. (Amended) The method of Claim 61, wherein said predetermined sites are determined [by] using secondary structure prediction or homology comparison.

63. (Amended) The method of Claim [54] 58, wherein said amino acid changes are introduced at random.

65. (Amended) The method of Claim 64, wherein [correctly folded variants are confirmed] said engineered protein is confirmed as having the conformation of said native protein by filter lift assay or ELISA.

67. (Twice amended) The method of Claim 58, wherein said nutritionally essential amino acids are increased to represent at least [10%] 20% of the total amino acid content of the protein.

68. (Amended) The method of Claim [54] 58, wherein said protein is vegetative storage protein.

69. (Amended) A method for altering the amino acid composition of a native protein of interest, said method comprising introducing amino acid changes into said protein to create an engineered protein having increased nutritional value, wherein:

a) said amino acid changes increase levels of at least one nutritionally essential amino acid in the engineered protein;

b) said nutritionally essential amino acid or nutritionally essential amino acids are increased to represent at least 10% of the total amino acid content of the engineered protein;

c) said engineered protein [having] has the conformation of the native protein;

d) [wherein] said conformation of the engineered protein is confirmed by binding said engineered protein with a set of interacting molecules capable of binding with the native protein[,]; and

e) [wherein] said molecules recognize native conformation.

72. (Amended) The method of Claim 69 wherein said interacting molecules are [dimerizing proteins] proteins that form homodimers or heterodimers with said native protein of interest.

75. (Twice amended) The method of Claim [74] 69, wherein at least one of said at least one nutritionally essential amino acid or nutritionally essential amino acids is methionine.

80. (Amended) The method of Claim 79, wherein [correctly folded variants are confirmed] said engineered protein is confirmed as having the conformation of said native protein by filter lift assay or ELISA.

82. (Twice amended) The method of Claim [73] 69, wherein said nutritionally essential amino acids are increased to represent at least [10%] 20% of the total amino acid content of the protein.

97. (Amended) A method for altering the amino acid composition of a vegetative storage protein, said method comprising introducing amino acid changes into said protein to create an engineered protein, wherein:

a) said amino acid changes alter the amino acid content of said protein by at least 10%;

b) said engineered protein [having] has the conformation of a native vegetative storage protein [wherein]; and

c) said conformation of the engineered protein is confirmed by binding with a panel of monoclonal antibodies which recognize the native protein conformation and are capable of binding said native vegetative storage protein [, and wherein said antibodies recognize native conformation].

99. (Twice amended) The method of Claim [98] 97, wherein said [nutritionally essential amino acids are selected from the group consisting of methionine, tryptophan, lysine, valine, phenylalanine, isoleucine, leucine, threonine and cysteine] amino acid changes comprise substitutions rather than deletions or additions.

105. (Amended) The method of Claim 104, wherein [correctly folded variants are confirmed] said engineered protein is confirmed as having the conformation of said native protein by filter lift assay or ELISA.

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nathic d., prognathi n d., dilatation of the pyloric end of the ach greater than that of the fundus, giving a protruding ap- ance in the roentgen-ray picture.

the st mach, distention of the stomach with retained secre- , food, and/or gas due to obstruction, ileus, or denervation; d also *gastric d.*

a·t r (dil″ə-ta′tər) [L.] 1. that which dilates, as a muscle. 2. NA native for *musculus dilator.*

ion (di-la′shən) 1. the act of dilating or stretching. 2. dilata-

al d., the expansion or stretching of a cavity or orifice by is f a finger.

or (di-la′tər) 1. [NA] a general term for a structure (muscle) dilates. 2. an instrument used in enlarging an orifice or canal by ching.

d., an instrument for dilating or stretching the anal sphincter.

orn's d., a metal dilator used to stretch the cardioesophageal n in cardiospasm.

r's d's, a series of bougies of varying sizes for dilating the m uteri.

oann's d., a metallic, expandable urethral dilator.

ugeal d., a bougie-like instrument which is used for distending a ised larynx.

'ris, the alar part of the nasal muscle; see *partes transversa et s musculi nasalis.*

matic d., a dilator for esophageal strictures, consisting of a le leading to an inflatable bag that straddles the stricture.

k d., an expandable rubber-covered metal frame used to dilate ardioesophageal region.

did (di-law′did) trademark for preparations of hydromor- e hydrochloride.

a·nus (di″lə-ka′nəs) [*di-* + Gr. *lekanē* a dish] dipygus.

id·i·dae (dil″ə-pid′i-de) a family of medium-sized or small vorms of the order Cyclophyllidea, subclass Cestoda, which itize mammals, birds, and snakes. The genus *Dipylidium* is of cal importance.

·zem hy·dro· hlo·ride (dil-ti′ə-zəm) a calcium channel er that acts as a vasodilator; used in the treatment of angina ris and hypertension.

abbreviation for L. *dilu′culo,* at daybreak.

nt (dil′u-ənt) [L. *diluere* to wash] 1. diluting. 2. an agent that s r renders less potent or irritant.

abbreviation for L. *dilu′tus,* diluted.

on (di-loo′shən) 1. the art or process of diluting or the state ing diluted. 2. a diluted or attenuated medicine. 3. in homeo- , the diffusion of a given quantity of a medicinal agent in ten or undred times the same quantity of water.

ling d., a serial dilution in which the dilution in each tube is l that of the preceding tube.

gen d., th addition of nitrogen to inspired air to lower its n tension, producing an alveolar oxygen tension equal to a ed oxygen pressure.

: d., a set of dilutions in a mathematical sequence. In microbio- l technique, serial dilutions are used to obtain a culture plate ields a countable number of separate colonies. From this, a lation of viable cells in the original suspension can be made, as ny picked for pure culture.

bbreviation for L. *dimid′ius,* one half.

·tig·amoe·ba (di-mas″tig-ə-me′bə) *Naegleria.*

a·dan (di-mef′ə-dān) chemical name: *N,N*-dimethyl-3- 1-indanamine; an analgesic, $C_{17}H_{19}N$.

il·c n A (di″mə-fil′kon) a contact lens material (hydro-).

line hy·dro·chlo·ride (di-mef′lēn) chemical name: methylamino)methyl]-7-methoxy-3-[methyl-2-phenyl-4*H*-1- pyran-4-one] hydrochloride; a respiratory stimulant, NO_3·HCl.

·lu·min (di-meg′loo-mēn) any salt containing two meglu- nolecules.

ia (di-me′le-ə) [*di-* + Gr. *melos* limb] a developmental anom- aracterized by duplication of a limb.

us (di-me′ləs) a fetus exhibiting dimelia.

·hy·dri·nat (di″mən-hi′drī-nāt) [USP] an antiemetic, used treatment of motion sickness and in other conditions in nausea may be a feature, administered orally.

·sion (dī-men′shən) a numerical expression, in appropri- ate units, of a linear measurement of an object, such as an organ body part.

vertical d., the distance between two points, measured perpendi lar to the horizontal. In prosthodontics, the length of the face de mined by the distance of separation of the jaws. See *contact verti d., postural vertical d.,* and *vertical d.*

vertical d., contact, vertical d., occlusal, the lower face height w the teeth in centric occlusion.

vertical d., postural, the vertical face height when the mandible suspended in the postural resting position.

vertical d., rest, the lower face height measured from a chin po just below the nose, with the mandible in the rest position.

di·men·sion·less (dī-men′shən-ləs) denoting a numerical consta or variable that has no units of measurement.

di·mer (di′mər) 1. a compound formed by combination of two id tical simpler molecules. 2. a capsomer having two structural s units.

thymine d., two adjacent thymine residues linked together by covalent bond along a single polynucleotide of DNA, which m lead to inactivation of the DNA molecule. It results from exposure ultraviolet radiation and may be reversed by photoreactivation.

di·mer·cap·rol (di″mər-kap′rol) [USP] a metal complexing age used as an antidote to poisoning by arsenic, gold, and mercury, a sometimes other metals, administered intramuscularly. It has al been used in the treatment of hepatolenticular degeneration.

di·mer·ic (di′mər-ik) exhibiting the characteristics of a dimer.

dim·er·ous (dim′ər-əs) [*di-* + Gr. *meros* part] made up of two par

di·me·tal·lic (di″mə-tal′ik) containing two atoms or equivalents o metallic element in the molecule.

Di·me·tane (di′mə-tān) trademark for preparations of brompheni amine maleate.

di·meth·i·cone (di-meth′i-kōn) 1. a silicone oil consisting of methylsiloxane polymers with viscosities from 0.65 to 3,000,0 centistokes at 25°C. The term is used with a numeric suffix whi indicates the approximate viscosity of the various grades in cen stokes, e.g., the viscosity of dimethicone 200 in centistokes is 190 210. Dimethicones are used as ingredients of ointments and oth preparations for topical application to protect the skin agai water-soluble irritants. 2. simethicone.

d. 350, a grade of dimethicone having a viscosity of approximate 350 in centistokes at 25° C; a prosthetic aid for soft tissues.

activated d., simethicone.

di·me·thin·dene mal·e·ate (di″mə-thin′dēn) an antihistamin administered orally.

di·me·thi·so·quin hy·dro·chlo·ride (di″mə-thi′so-kwin) a lo anesthetic applied topically to relieve pain, itching, and burning the skin.

di·me·this·ter·one (di″mə-this′tər-ōn) an orally effective proge tin, having actions and uses similar to those of progesterone; us alone or as the progestin component in combination with ethi estradiol as an oral contraceptive.

di·me·thox·a·nate hy·dro·chlo·ride (di″mə-thok′sə-nāt) che cal name: 10*H*-phenothiazine-10-carboxylic acid 2-[2-(dimeth amino)ethoxy]ethyl ester hydrochloride; an antitussiv $C_{19}H_{22}N_2O_5S$·HCl.

2,5-di·me·thoxy-4-meth·yl·am·phet·amine (di″mə-thok meth″əl-am-fet′ə-mēn) a hallucinogenic compound derived f amphetamine; abbreviated DOM and popularly called STP.

3,4-di·me·thoxy·phen·yl·eth·yl·amine (di″mə-thok″se-fen eth″əl-am′ən) a substance characteristically found in the urine schizophrenics; abbreviated DMPE.

di·meth·yl·amine (di-meth″əl-am′ən) a gaseous and liquid p maine, from decaying gelatin, decomposing yeast, rotten fish, et

***p*-di·meth·yl·a·mi·no·azo·ben·zene** (di-meth″əl-ə-me″no-a ben′zēn) a dicyclic carcinogenic compound used as an indicator tests for and in Ehrlich's aldehyde reaction to detect urobilinoge It has a pH range of 2.9 to 4, being red at 2.9 and yellow at 4. Call also *butter yellow* and *methyl yellow.*

di·meth·yl·ami·no·pro·pio·ni·tril (DMAPN) (di-meth″əl me″no-pro″pe-o-ni′tril) a colorless water-soluble liquid used in th manufacture of polyurethane foam; workers with excessive exp sure to it are prone to urologic and neurologic disorders.

Di·m th·y·lane (di-meth′ə-lān) trademark for a preparation of pr moxolane.

di·meth·yl·ar·sin (di-meth″əl-ahr′sēn) cacodyl hydride.

meth·yl·ar·sin

-di·m th·yl·b) 9,10-dimeth lic aromatic h h of carbonace tabolic activat ic effect; it is Abbreviated

meth·yl·b n·ze

-di·meth·yl·b erivative of benz is an unusual olety forms the

meth·yl·car·ba less liquid used harmaceuticals, ly carcinogenic.

meth·yl car·ba an insect repe aled.

meth·yl· ar·bi·

meth·yl· th·yl· yrrole obtained

-di·m th·yl·gl ediate occurrin etabolism of ch

meth·yl·gly·cin l′dro-jən-ās) [EC he oxidoreducta ion of *N,N*-dimeth ine. It requires a lavin moiety to el in the mitochondr

meth·yl·ke·t n

meth·yl·ni·tro·s ethylamine.

meth·yl·ph ·na enic and weakly

meth·yl phthal· iquid, the normal epellent.

meth·yl sul·fat dhesives and as everely irritant to enic.

meth·yl sulf·ox ractically odorles iquid, it is a powe trated hydrocarb tances. Its biologi nd animal tissues een used investig natory agent and a ubstances. It is av ation into the blac lated DMSO.

m th·yl·tryp·ta· ubstance derived (Benth.) Macb enth.) which is n ndles. Abbreviated

m·i·nu·ti n (dim″ ubstance.

m·m r's keratiti halmologist, 1855–

mo·cil·lin (di-mo- ethicillin.

mor·phic (di-mor′

mor·phism (di-mo of having or existing yeasts. See also *dys*

physical d., the pro line or allotropic for

sexual d., physica ender.

…l (ho-mak'se-əl) having axes of the same length.

…syndrome (ho'mānz) [Ernst Alexander *Homén*, Finnish …1851–1926] postconcussional syndrome.

homoeo( )-, homoi( )- [Gr. *homoios* like, resembling] a …form denoting sameness or similarity.

…x (ho'me-o-boks") any of a class of highly conserved …ences, approximately 180 base pairs long, encoding a pro…in involved in binding to DNA; it was named for its initial …as a *Drosophila* locus important in homeotic mutation, …occurs in humans and is usually found in genes involved …ntrol of development.

…rome (ho'me-o-krōm") [*homeo-* + Gr. *chrōma* color] …ith mucin stains after formol-bichromate fixation; applied …n serous cells of the salivary glands. Cf. *tropochrome*.

…-ne-sis (ho"me-o-kī-ne'sis) [*homeo-* + Gr. *kinēsis* mo… stage of meiosis in which the daughter cells receive equal …nd kinds of chromatin.

…orphous (ho"me-o-mor'fəs) [*homeo-* + Gr. *morphē* …like form and structure.

…-teo-plas-ty (ho"me-o-os"te-o-plas'te) [*homeo-* + Gr. *os*… + *plassein* to mold] the grafting of bone from one indi… another within the same species.

…ath (ho'me-o-path) homeopathist.

…ath-ic (ho"me-o-path'ik) pertaining to homeopathy.

…-thist (ho"me-op'ə-thist) one who practices homeo…m

…-thy (ho"me-op'ə-the) [*homeo-* + Gr. *pathos* disease] a …of therapeutics founded by Samuel Hahnemann (1755–…which diseases are treated by drugs which are capable of …g in healthy persons symptoms like those of the disease to …d, the drug being administered in minute doses. Cf. *allo-*

…a-sia (ho"me-o-pla'zhə) [*homeo-* + Gr. *plassein* to form] …ition of new tissue like that adjacent to it and normal to the …

…as-tic (ho"me-o-plas'tik) 1. resembling in structure the …parts. 2. pertaining to, characterized by, or stimulating …sia.

…re-sis (ho"me-o-re'sis) [*homeo-* + Gr. *rhein* to flow] the …to maintain a biological process, as a growth process, …articular pathway despite the operation of factors tending …

…is (ho"me-o'sis) [Gr. *homoiōsis* likeness, resemblance] …ation of a body part having the characteristics normally …related part at a different body site.

…a-sis (ho"me-o-sta'sis) [*homeo-* + Gr. *stasis* standing] a …to stability in the normal body states (internal environ… the organism. It is achieved by a system of control mecha…ulated by negative feedback; e.g., a high level of carbon …n extracellular fluid triggers increased pulmonary ventila…ch in turn causes a decrease in carbon dioxide concentra…

…at-ic (ho"me-o-stat'ik) pertaining to homeostasis.

…ther-a-py (ho"me-o-ther'ə-pe) [*homeo-* + Gr. *therapeia* …] treatment or prevention of disease with a substance …but not the same as the causative agent of the disease.

…therm (ho'me-o-thərm") [*homeo-* + Gr. *thermē* heat] 1. an …hat exhibits homothermy; a so-called warm-blooded ani…posed to a poikilotherm. 2. endotherm.

…ther-mal (ho"me-o-thər'məl) [*homeo-* + Gr. *thermē* heat] …rmic.

…ther-mic (ho"me-o-thər'mik) 1. pertaining to or character… homeothermy (def. 1). 2. endothermic (def. 2).

…ther-mism (ho"me-o-thər'miz-əm) homeothermy.

…ther-my (ho'me-o-thər"me) the maintenance of a constant …mperature despite changes in the environmental tempera… *poikilothermy* (defs. 1 and 2).

…typ-ic, ho-meo-typ-i-cal (ho"me-o-tip'ik, ho"me-o-tip'i-…meo- + Gr. *typos* type] resembling the normal or usual type.

…ic (hōm-ər'jik) [*hom (o)-* + Gr. *ergon* work] having the …ect; said of two drugs each of which produces the same …ect.

…e (hom'i-sīd) [L. *homo* man + *caedere* to kill] the taking of …another individual.

h -mid-i-um (ho-mid'e-əm) a trypanosomicide used as the bromide and chloride salts in the treatment of infections with *Trypanosoma congolense* and *T. vivax* in cattle and horses. In biochemistry, it is usually called *ethidium*, and the bromide salt is used as fluorochrome in the detection of double-stranded nucleic acids.

hom-i-nal (hom'i-nəl) [L. *homo* man] pertaining to man; pertaining to human beings.

hom-i-nid (hom'i-nid) 1. pertaining to the family of humans (Hominidae). 2. a living or extinct human or humanlike type.

Ho-min-i-dae (ho-min'i-de) [L. *homo* man + Gr. *eidos* resemblance] a family of primates (superfamily Hominoides, suborder Anthropoidea), including both modern man (*Homo sapiens*) and fossil hominids.

hom-in-i-nox-ious (hom"i-ne-nok'shəs) injurious to man.

hom-i-noid (hom'i-noid)) 1. pertaining to the Hominoidea. 2. a member of the Hominoidea.

Hom-i-noi-dea (hom"i-noi'de-ə) [L. *homo* man + Gr. *oeidos* likeness] a superfamily of primates (suborder Anthropoidea), including the families Pongidae (anthropoid apes) and Hominidae (man, both modern and extinct).

homme (um) [Fr.] man.
h. rouge (roozh') [Fr. "red man"], a stage in mycosis fungoides in which the red plaques become infiltrated and coalesce over a wide area of the body.

Ho-mo (ho'mo) [L. *man*] the genus of primates (family Hominidae, superfamily Hominoides) that includes humans (*H. sapiens*) and fossil hominids.

hom(o)- [Gr. *homos* same] 1. a combining form meaning the same. 2. a prefix in chemical names indicating the addition of one CH_2 group to the main compound.

ho-mo-ar-te-re-nol hy-dro-chlo-ride (ho"mo-ahr"tə-re'nol) norde…frin hydrochloride.

ho-mo-bio-tin (ho"mo-bi'o-tin) a homologue of biotin having an additional CH_2 group in the side chain and acting as a biotin antagonist.

ho-mo-body (ho'mo-bod"e) an antibody with an idiotypic determinant that is stereochemically similar to the epitope on the antigen against which the antibody was originally directed; it is therefore able to mimic the behavior of the antigen.

ho-mo-car-no-sin-ase (ho"mo-kahr'no-sī-nās) former name for an enzyme activity now believed to be part of the serum isozyme of X-His dipeptidase (carnosinase).

ho-mo-car-no-sine (ho"mo-kahr'no-sēn) a dipeptide consisting of γ-aminobutyric acid and histidine; in humans it is found in the brain but not in other tissues.

ho-mo-car-no-sin-o-sis (ho"mo-kahr-no"sī-no'sis) an inherited aminoacidopathy characterized by accumulation of homocarnosine in cerebrospinal fluid and the brain but not in plasma or urine, accompanied by carnosinuria. Progressive spastic paraplegia, mental deterioration, and retinal pigmentation may be sequelae. The disorder is due to deficiency of the serum isozyme of X-His dipeptidase, but its relationship to serum carnosinase deficiency (q.v.) has not been elucidated.

ho-mo-cen-tric (ho"mo-sen'trik) [*homo-* + Gr. *kentron* center] having the same center or focus.

ho-moch-ro-nous (ho-mok'ro-nəs) [*homo-* + Gr. *chronos* time] occurring at the same age in successive generations.

ho-mo-cin-cho-nine (ho"mo-sin'ko-nin) an alkaloid, $C_{19}H_{22}ON_2$, from cinchona, isomeric with cinchonine.

ho-mo-clad-ic (ho"mo-klad'ik) [*homo-* + Gr. *klados* branch] formed between small branches of the same artery; said of such an anastomosis.

ho-mo-cyc-lic (ho"mo-sik'lik) having or pertaining to a closed chain or ring formation which includes only atoms of the same element.

ho-mo-cys-te-ine (ho"mo-sis'-tēn) a sulfur-containing amino acid homologous with cysteine and produced by demethylation of methionine. It can serve as an intermediate in the biosynthesis of cysteine from methionine via cystathionine or can be remethylated to methionine.

ho-m -cys-te-ine-tet-ra-hy-dro-fo-late meth-yl-trans-fer-as (ho"mo-sis'teēn tet"rə-hī"dro-fo'lāt meth"əl-trans'fər-ās) 5-methyltetrahydrofolate-homocysteine *S*-methyltransferase.

h -mo-cys-tin (ho"mo-sis'tēn) a disulfide homologous with cystine and formed by oxidation and subsequent condensation of two molecules of homocysteine. It is a source of sulfur in the body.

er·ax·i·al (het″ər-ak′se-əl) [*heter-* + *axis*] having axes of unequal length.

er·e·cious (het″ər-e′shəs) [*heter-* + Gr. *oikos* house] living upon one host in one stage or generation and upon another in the next.

er·e·cism (het″ər-e′siz-əm) the state of being heterecious.

er·er·gic (het″ər-ər′jik) [*heter-* + Gr. *ergon* work] having different effects; said of two drugs one of which produces a particular effect and the other does not.

er·es·the·sia (het″ər-es-the′zhə) [*heter-* + Gr. *aisthēsis* perception] variation in the degree of cutaneous sensibility on adjoining areas of the body surface.

ter(o)- [Gr. *heteros* other, different] a combining form meaning other, different, or abnormal, or denoting relationship to another.

ero·ag·glu·ti·na·tion (het″ər-o-ə-gloo″tĭ-na′shən) agglutination of particulate antigens (on cells or adsorbed on inert carrier particles) of one species by agglutinins derived from organisms of another species.

ero·ag·glu·ti·nin (het″ər-o-ə-gloo′tĭ-nin) an agglutinin with reactive specificity for particulate antigen(s) in one or more species other than the species in which it originates.

ero·al·bu·mose (het″ər-o-al′bu-mōs) a form of hemialbumose that is not soluble in water, but is soluble in hydrochloric acid and sodium chloride solutions.

ero·al·bu·mos·uria (het″ər-o-al″bu-mōs-u′re-ə) the presence of heteroalbumose in the urine.

ero·an·ti·body (het″ər-o-an″tĭ-bod′e) an antibody specific for antigens originating in a species other than that of the antibody producer.

ero·an·ti·gen (het″ər-o-an′tĭ-jən) an antigen originating in a species different from, and therefore foreign to, the antibody producer.

ero·at·om (het″ər-o-at′om) [*hetero-* + *atom*] any atom in an organic compound other than carbon or hydrogen.

ero·bil·har·zia (het″ər-o-bil-hahr′zhə) a genus of schistosomes that parasitize mammals, including human beings.
H. america′na, a species whose cercariae may cause a nonpatent visceral schistosomiasis in man.

ero·blas·tic (het″ər-o-blas′tik) [*hetero-* + Gr. *blastos* germ] having origin in different kinds of tissue.

ero·cel·lu·lar (het″ər-o-sel′u-lər) composed of cells of different kinds.

ero·cen·tric (het″ər-o-sen′trik) [*hetero-* + L. *centrum* center] made up of rays that neither are parallel nor meet in one point; said of a ray of light.

ero·ceph·a·lus (het″ər-o-sef′ə-ləs) [*hetero-* + Gr. *kephalē* head] a fetus with two unequal heads.

ero·chi·ral (het″ər-o-ki′rəl) [*hetero-* + Gr. *cheir* hand] reversed as regards right and left, but otherwise the same in form and size, as the hands.

ero·chro·ma·tin (het″ər-o-kro′mə-tin) [*hetero-* + *chromatin*] that state of chromatin in which it is dark-staining and tightly coiled, forming irregular clumps (karyosomes) or Barr bodies in the nuclei of cells in interphase, or stains densely in certain areas of mitotic chromosomes. Cf. *euchromatin*.
constitutive h., the chromatin in regions of the chromosomes that are invariably heterochromatic, located in secondary constrictions of chromosomes 1, 9, and 16, the distal end of the long arm of the Y chromosome, and centromeric and telomeric regions; it contains highly repetitive sequences of DNA that are genetically inactive, and serves as a structural element of the chromosome. See also *C banding*, under *banding*.
facultative h., the chromatin in regions of the chromosomes that become heterochromatic in certain cells and tissues; e.g., it makes up the inactive X chromosome in female somatic cells.

ero·chro·ma·tin·iza·tion (het″ər-o-kro″mə-tin-ĭ-za′shən) 1. the condensation of euchromatin into heterochromatin. Called also *heterochromatization*. 2. lyonization.

ero·chro·ma·ti·za·tion (het″ər-o-kro″mə-tĭ-za′shən) 1. heterochromatinization. 2. lyonization.

ero·chro·ma·to·sis (het″ər-o-kro″mə-to′sis) heterochromia.

ero·chro·mia (het″ər-o-kro′me-ə) [*hetero-* + Gr. *chrōma* color + *-ia*] diversity of color in a part or parts that should normally be of one color.
i. i′ridis, difference of color in the two irides, or in different areas of the same iris.

h t· ro·chro·m ·s me (het″ər-o-kro′mo-sōm) [*hetero-* + *chromosome*] a sex chromosome.

h t·ero·chro·m us (het″ər-o-kro′məs) marked by diversity of color; exhibiting heterochromia.

het· ro·chro·nia (het″ər-o-kro′ne-ə) [*hetero-* + Gr. *chronos* time + *-ia*] 1. the formation of parts or tissues, or the occurrence of a phenomenon, at an unusual time. Cf. *synchronia* (def. 2). 2. a difference in the rate or time of occurrence between two processes.

het·ero·chron·ic (het″ər-o-kron′ik) [*hetero-* + Gr. *chronos* time] 1. pertaining to or characterized by heterochronia. 2. denoting different ages or stages of development, as between the excised organ and the implanted organ in transplantation procedures.

het·er·och·ro·nous (het″ər-ok′ro-nəs) heterochronic.

het·er·och·tho·nous (het″ər-ok′tho-nəs) [*hetero-* + Gr. *chthōn* a particular land or country] originating in a region other than that in which it is found. Cf. *autochthonous* (def. 1).

het·ero·chy·lia (het″ər-o-ki′le-ə) the sudden varying of the gastric secretion from normal acidity to hyperacidity or anacidity.

het·ero·clad·ic (het″ər-o-klad′ik) [*hetero-* + Gr. *klados* branch] indicating an anastomosis between terminal branches from different arteries.

het·ero·crine (het′ər-o-krēn) [*hetero-* + Gr. *krinein* to separate] secreting more than one kind of matter.

het·er·oc·ri·sis (het″ər-o-kri′sis) [*hetero-* + Gr. *krisis* division] an abnormal crisis with unusual timing and symptoms.

het·ero·cyc·lic (het″ər-o-sīk′lik) [*hetero-* + Gr. *kyklos* circle] having or pertaining to a closed chain or ring formation which includes atoms of different elements.

het·er·o·cy·to·trop·ic (het″ər-o-si″to-trop′ik) [*hetero-* + *cyto-* + Gr. *tropos* a turning] having an affinity for cells of different species; see under *antibody*.

Het·er·od·era rad·i·cic·o·la (het″ər-od′ə-ra rad″ĭ-sik′o-lə) a nematode parasitic on the common root vegetables, such as radishes, carrots, turnips, potatoes, etc., as well as on celery. When infested vegetables are eaten, ova of the parasite may appear in the stools and must be distinguished from those of true parasites.

het·ero·der·mic (het″ər-o-dər′mik) [*hetero-* + Gr. *derma* skin] denoting a skin graft taken from a member of another species. See *dermatoheteroplasty*.

het·ero·des·mot·ic (het″ər-o-des-mot′ik) [*hetero-* + Gr. *desmos* a bond] joining dissimilar parts of the central nervous system; see under *fiber*.

het·ero·did·y·mus (het″ər-o-did′ə-məs) heterodymus.

het·ero·di·mer (het″ər-o-di′mər) [*hetero-* + *dimer*] a dimer consisting of unlike subunits.

het·ero·dont (het′ər-o-dont) [*heter-* + Gr. *odous* tooth] having teeth of different types, such as incisors and molars.

Het·ero·dox·us (het″ər-o-dok′səs) a genus of insects of the order Mallophaga, the biting lice. *H. longitarsus* is parasitic on kangaroos, wallabies, and sometimes dogs in Australia. *H. spiniger* is parasitic on coyotes and wolves in the New World, and may also infest dogs.

het·er·od·ro·mous (het″ər-od′ro-məs) [*hetero-* + Gr. *dromos* running] moving, acting, or arranged in the opposite direction.

het·er·od·y·mus (het″ər-od′ə-məs) [*hetero-* + Gr. *didymos* twin] a fetus with a second head, neck, and thorax attached to the thorax.

het·er·oe·cious (het″ər-o-e′shəs) requiring two or more hosts to complete the life cycle; said of certain fungi and insects. Cf. *autoecious*.

het·ero·erot·i·cism (het″ər-o-ə-rot′ĭ-siz-əm) sexual feeling directed toward another person; cf. autoeroticism.

het·ero·er·o·tism (het″ər-o-er′o-tiz-əm) heteroeroticism.

het·ero·fer·men·ta·tion (het″ər-o-fər″mən-ta′shən) fermentation producing more than one major product; the term is often used as a synonym of heterolactic fermentation (q.v.).

het·ero·fer·ment·er (het″ər-o-fər-men′tər) a microorganism that exhibits heterofermentation.

het· ro·gam· te (het″ər-o-gam′ēt) a gamete of different size and structure than the one with which it unites.

het·ero·ga·met·ic (het″ər-o-gə-met′ik) pertaining to the sex that produces gametes of different kinds, in terms of their sex chromosomes. In human beings the male, who possesses X-bearing and Y-bearing sperm, is the heterogametic sex.

het·er ·gam·e·ty (het″ər-o-gam′ə-te) the production of un-

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The Soybean Vegetative Storage Proteins VSPα and VSPβ Are Acid Phosphatases Active on Polyphosphates*

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The soybean vegetative storage protein genes (*vspA*, and *vspB*) are regulated in a complex manner developmentally and in response to external stimuli such as wounding and water deficit. The proteins accumulate to almost one-half the amount of soluble leaf protein when soybean plants are continually depodded and have been identified as storage proteins because of their abundance and pattern of expression in plant tissues. We have shown that purified VSP homodimers (VSPα and VSPβ) and heterodimers (VSPα/β) possess acid phosphatase activity (α = 0.3–0.4 units/mg; β = 2–4 units/mg; α/β = 7–10 units/mg). Specific activities w re determined by monitoring *o*-carboxyphenyl phosphate (0.7 mM) cleavage at pH 5.5 (VSPα) or pH 5.0 (VSPα/β and VSPβ) in 0.15 M sodium acetate buffer at 25 °C. These enzymes are active over a broad pH range, maintaining greater than 40% of maximal activity from pH 4.0 to 6.5 and having maximal activity at pH 5.0–5.5. They are inactivated by sodium fluoride, sodium molybdate, and heating at 70 °C for 10 min. These phosphatases can liberate P_i from several different substrates, including napthyl acid phosphate, carboxyphenyl phosphate, sugar-phosphates, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, phosphoenolpyruvate, ATP, ADP, PP_i, and short chain polyphosphates. VSPα/β cleaved phosphoenolpyruvate, ATP, ADP, PP_i, and polyphosphates most efficiently. Apparent K_m and V_{max} values at 25 °C and pH 5.0 were 42 μM and 2.0 μmol/min/mg, 150 μM and 4.2 μmol/min/mg, and 420 μM and 4.1 μmol/min/mg, for tetrapolyphosphate, pyrophosphate, and phosphoenolpyruvate, respectively.

In 1982 and 1983, Wittenbach (1–3) described proteins that accumulated in leaves of soybean when the plants were continually depodded. The accumulation of these proteins is remarkable because after weeks of depodding, they represented over 45% of the soluble protein in leaves. Franceschi and co-workers (4–6) determined that the proteins are localized in vacuoles of bundle sheath and paraveinal mesophyll (PVM)[1] cells of leaves. Later studies showed that these proteins also accumulate in epidermal cells (7). The PVM cells play an important role in transferring amino acids and carbon between the vascular system and mesophyll cells of the leaf. The accumulation of the proteins in response to removal of reproductive sinks and their localization in vacuoles of transfer cells led Wittenbach to name them vegetative storage proteins (VSPα, and VSPβ). More recent studies have shown that *vsp* mRNA and protein accumulate in young leaves, stems, flowers, pods, and the cotyledons of germinating seedlings, but are rare in seeds and roots (8–12). The expression of the *vsp* is decreased when plants are nitrogen-limited (13). In contrast, *vsp* mRNA accumulation is stimulated by carbon sources such as sucrose and by the plant growth regulator jasmonic acid (14). It is noteworthy that other soybean proteins (*e.g.* a lipoxygenase) with similar expression patterns and intracellular localization have been described (15, 16).

VSP accumulation is modulated developmentally and by environmental factors (8–14, 17). Bozarth *et al.* (17) first reported that VSPα accumulated in cell wall fractions in soybean seedlings exposed to water deficit. Later studies showed that *vsp* mRNA and protein accumulate in seedlings in response to water deficit (8). When water is withheld from older plants, mRNAs accumulate in internodes and intermediate age leaves (12). The accumulation of VSP under these conditions has been attributed to decreased growth coupled with continued transport of amino acids and carbon to growing regions (12). The VSP are also induced in wounded plants, although their function in this response is unknown (7, 12). The induction of the genes in wounded tissue appears to result from wound-induced accumulation of jasmonic acid or its methyl ester, methyl jasmonate. This idea is consistent with the stimulation of *vsp* expression by addition of jasmonic acid or methyl jasmonate to cell cultures or plants (12–14, 18, 19). In addition, inhibitors of lipoxygenase, which should limit jasmonic acid biosynthesis in wounded tissue, block wound-induced accumulation of the VSP (13).

The VSP are glycoproteins that are 80% identical in amino acid sequence (8, 9). However, the isoelectric points of VSPα and VSPβ are 8.6 and 5.8, respectively (8). Based on cDNA sequences (8), the predicted protein molecular masses are 25.1 (α) and 25.3 kDa (β), and based on chemical analysis, the mature proteins are approximately 3% carbohydrate (20). The VSP have been reported to migrate on SDS-PAGE gels with apparent molecular masses of 25–28 kDa for VSPα and 27–31 kDa for VSPβ (1–4, 7–11, 17–21). The native form of VSP in soybean leaves is α_2, αβ, and β_2 dimers (20, 21).

Recently, Williamson and Colwell (22), Aarts *et al.* (23), and Erion *et al.* (24) reported the isolation of an acid phosphatase from tomato that is encoded by *Aps-1*. These authors noted that portions of the acid phosphatase-1 sequence were similar to VSP sequences, suggesting that the vegetative storage proteins might have acid phosphatase activity. We have investigated this possibility and report that VSPα and

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[1] The abbreviations used are: PVM, paraveinal mesophyll; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VSP, vegetative storage proteins; ConA, concanavalin A; PEP, phosphoenolpyruvate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

VSPβ are acid phosphatases that have substrate preference for polyphosphates, pyrophosphate, and PEP.

EXPERIMENTAL PROCEDURES

Supplies—Ammonium sulfate, concanavalin A-Sepharose (ConA) affinity resin, the Mono Q column, and the fast protein liquid chromatography system are products of Pharmacia LKB Biotechnology Inc. Electrophoresis equipment and chemicals were purchased from Bio-Rad. Nitrocellulose (0.45 μm) was from Schleicher & Schuell, and all other chemicals were purchased from Sigma.

Plant Material—Soybean (*Glycine max* Merr cv Williams 82) seedlings were grown at 27 °C in the dark at high humidity for hypocotyl tissue or in constant light (350 microeinsteins m^{-2} s^{-1}) at 25 °C for leaf tissue. Hypocotyl tissue harvested for protein purification was the growing hook and elongating region of 3-day-old seedlings, and young leaves were initial trifoliates from 12-day-old seedlings.

Protein Extraction and Isolation—VSPα and VSPβ were purified by a modification of the procedure described by Spilatro and Anderson (20) with all manipulations performed at 4 °C or colder. Hypocotyls or leaves were harvested and immediately frozen in liquid nitrogen. Tissue was stored at −80 °C until extraction. Extractions were carried out by grinding tissue in liquid nitrogen and then adding buffer (50 mM Tris/HCl (pH 7.5), 5 mM EDTA, 30 mM ascorbic acid, 10 mM dithiothreitol, 0.8 mM phenylmethylsulfonyl fluoride) at a ratio of 1:5 w/v. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 15,000 × *g* for 20 min. Finely ground ammonium sulfate was added to the supernatant sequentially to final concentrations of 1.7, 2.0, and 2.8 M. The 2.8 M pellet was resuspended in a minimal volume of 20 mM Tris/HCl (pH 7.5), 10 mM dithiothreitol, and dialyzed overnight against 2,000 volumes of the resuspension buffer. The dialysate was centrifuged at 14,000 × *g*, and the supernatant was applied, collected, and reapplied to a ConA column. The affinity column was washed with five column volumes of 50 mM sodium phosphate (pH 7.5), 200 mM sodium chloride to remove unbound protein, and elution of bound protein was achieved by washing the column with loading buffer containing 200 mM α-methyl-D-mannopyranoside. Fractions containing concentrated VSP were pooled and dialyzed against 20 mM Tris/HCl, 5 mM dithiothreitol. The dialysate was cleared by centrifugation, and VSP was purified with fast protein liquid chromatography using a Mono Q column.

Protein Concentration Determination—Protein concentrations were determined by the dye-binding method of Bradford (25) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis—Denaturing SDS-PAGE (12% acrylamide, acrylamide:bisacrylamide = 30:0.8) was performed by the method of Laemmli (26). Nondenaturing gel electrophoresis (8%) was performed as above, except that SDS and mercaptoethanol were not used in any buffers. Protein electroblotting and antibody detection of blotted proteins were performed as described (27) using alkaline phosphatase-conjugated secondary antibody (Bio-Rad). Polyacrylamide acid phosphatase activity gels (8% nondenaturing) were developed using conditions described previously (28).

Antibody Preparation—For preparation of polyclonal antibodies against VSP, fusion proteins were expressed in *Escherichia coli*. A portion of the open reading frame from cDNAs (8) encoding either VSPα (pKSH2, amino acids 195–254) or VSPβ (pKSH3, amino acids 196–254) on a *Bam*HI/*Cla*I fragment was fused in-frame to the 3′ end of a portion of the *trpE* gene of *E. coli* in the vector pATH 10 (kindly supplied by C. Dieckman, University of Arizona, Tucson). Transformation of *E. coli* strain RR1 with the recombinant plasmids and induction of the fusion proteins were performed as described (29). The fusion proteins were extracted and partially purified by precipitation with high salt (30) and further purified by SDS-PAGE on 12% acrylamide gels and electroelution of Coomassie-stained proteins (29). Female New Zealand rabbits were immunized by subcutaneous injection of protein solutions emulsified with equal volumes of Freund's adjuvant and boosted monthly before serum was collected after 4 months.

Acid Phosphatase Assays—Acid phosphatase activity of purified protein was quantitated using assay conditions described in the Worthington enzyme manual (Worthington Biochemical Corp., Freehold, NJ). Briefly, all assays were conducted using 0.15 M sodium acetate buffer at the pH described with 0.7 mM *o*-carboxyphenyl phosphate as the substrate. Increase in absorbance at 300 nm corresponding to the release of salicylic acid was monitored using a Beckman DU 64 spectrophotometer and the kinetics software compatible with this instrument. One unit of enzyme hydrolyzes 1 μmol of *o*-carboxyphenyl phosphate/min at 25 °C, pH 5.0 under the conditions specified above.

Assays to determine pH optimum were done as above, except different buffers were used at higher pH. The activity of VSPα and VSPβ homodimers and the VSPα/β heterodimer was determined in acetate (3.5–6.0), MES (5.5–6.5), and MOPS (6.5–7.5) buffers. All assays were duplicated, and activities at overlapping pH in different buffers were averaged. No difference in relative activity greater than 10% was observed at the same pH in different buffers.

Phosphatase Substrate Specificity—Release of inorganic phosphate from substrates was measured by the method of Taussky and Shorr (31). All assays were performed in duplicate at pH 5.0 or 5.5 and 25 °C. Cleavage of substrate was examined using 0.01–0.03 units/assay enzyme and 5 mM substrate. The release of phosphate in control samples (no enzyme) was measured and subtracted from corresponding enzyme-containing samples. Inorganic phosphate measured fell within the linear portion of the standard curve (0.2–40 μg) for this assay. The fraction of consumed substrate was always less than 15% of the total in each sample, indicating that initial velocities were measured.

K_m and V_{max} Determination—We determined apparent K_m and V_{max} values of purified hypocotyl VSPα/β for phosphoenolpyruvate, pyrophosphate, and tetrapolyphosphate using KinetAsyst software (Intellikinetics, State College, PA) created from programs detailed by Cleland (32). The rate of substrate cleavage by 0.01 units of VSPα/β was measured at substrate concentrations from 1 μM to 10 mM. Assays were performed at pH 5.0, 25 °C, for 5 min.

RESULTS

Sequence Comparison of Acid Phosphatase-1, VSPα, and VSPβ—VSPα and VSPβ have 45% overall amino acid sequence identity with acid phosphatase-1 (22–24). Several amino acid regions have a high degree of similarity among the three proteins (see Ref. 24). Acid phosphatase activity probably requires participation of histidyl groups (33). We noted that His^{124} (numbering for acid phosphatase-1) and His^{189} were conserved among the three proteins. In contrast, a Gly-Pro-Gly-Tyr motif located in acid phosphatase-1 (amino acids 75–78), characteristic of alkaline phosphatases (23), is not located in the VSP sequences. Acid phosphatase-1 shows affinity for ConA columns consistent with evidence that it is a glycoprotein (34). Similarly, VSPα and VSPβ are glycoproteins, and each contains a potential site for asparagine-linked glycosylation (amino acids 129–131 and 130–132; NST and NET (see Ref. 8 for numbering)). This site is not conserved in acid phosphatase-1, but a site nearby (amino acids 142–144; NGT) could represent a glycosylation site. Another difference between these proteins is that their isoelectric points (pI) vary widely (VSPα = 8.6, VSPβ = 5.8, acid phosphatase-1 = 4.7).

Purification of VSPα, VSPβ, and VSPα/β Dimers—The VSP were purified by ammonium sulfate precipitation and chromatography on ConA and Mono Q columns. The elution profiles for the Mono Q chromatography are shown in Fig. 1. We found that hypocotyls represent an enriched source of VSPβ and leaves an enriched source of VSPα. Therefore, VSPα/β and VSPβ were purified from hypocotyls as shown in the top chromatogram in Fig. 1, where VSPα/β elutes in the first indicated peak and VSPβ elutes in the second indicated peak. Hypocotyl VSPα/β and VSPβ were used for subsequent enzymes studies. The Mono Q elution profile shown in the *bottom panel* of Fig. 1 is of a leaf extract with VSPα eluting in the first indicated peak and VSPα/β eluting in the second indicated peak. The VSPα derived from this purification was used in subsequent enzyme studies; however, the VSPα/β fraction from leaf contained contaminating proteins and was not used in any enzyme analysis. The stained gel shown at the top of the elution profiles further illustrates that the different VSP dimers are readily separated by anion-exchange chromatography (Mono Q). These data are consist-

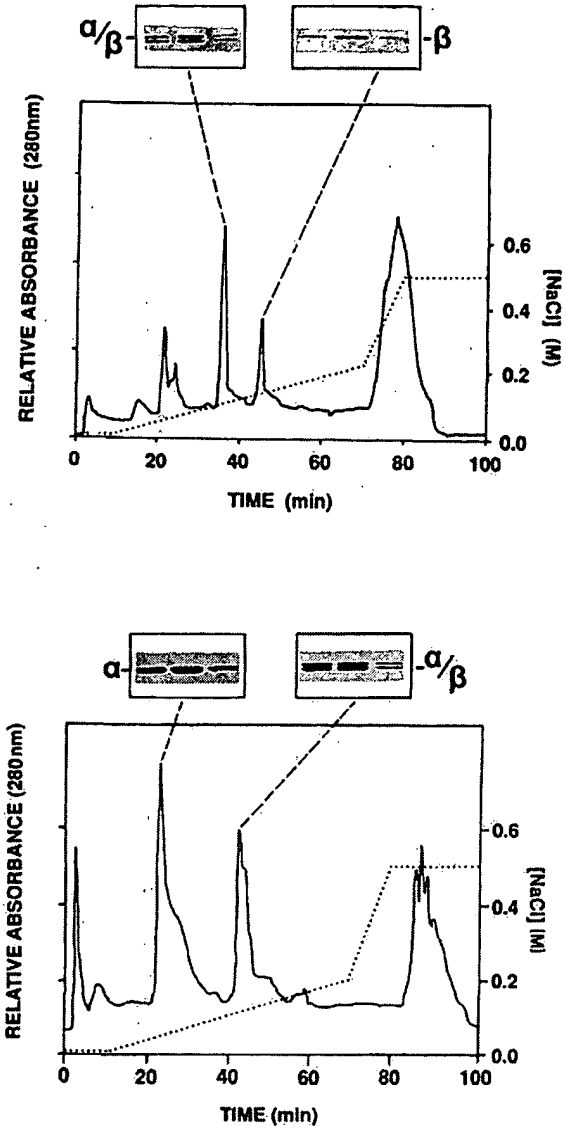

FIG. 1. **Elution profiles of soybean tissue extracts chromatographed on a Mono Q column.** Chromatograms of the final step in VSP purification. The *upper panel* shows the profile obtained when hypocotyl proteins are eluted from a Mono Q column, and the *lower panel* shows the profile obtained when leaf proteins are eluted from a Mono Q column. *Insets* above each chromatogram are photographs of Coomassie Blue-stained gel sections of the three fractions aligned directly with the indicated absorbance peak. VSPα/VSPβ heterodimers and VSPβ homodimers were purified from hypocotyl tissue. VSPα homodimers were purified from leaf tissue. The *dotted lines* indicate the concentration of NaCl in the elution buffer.

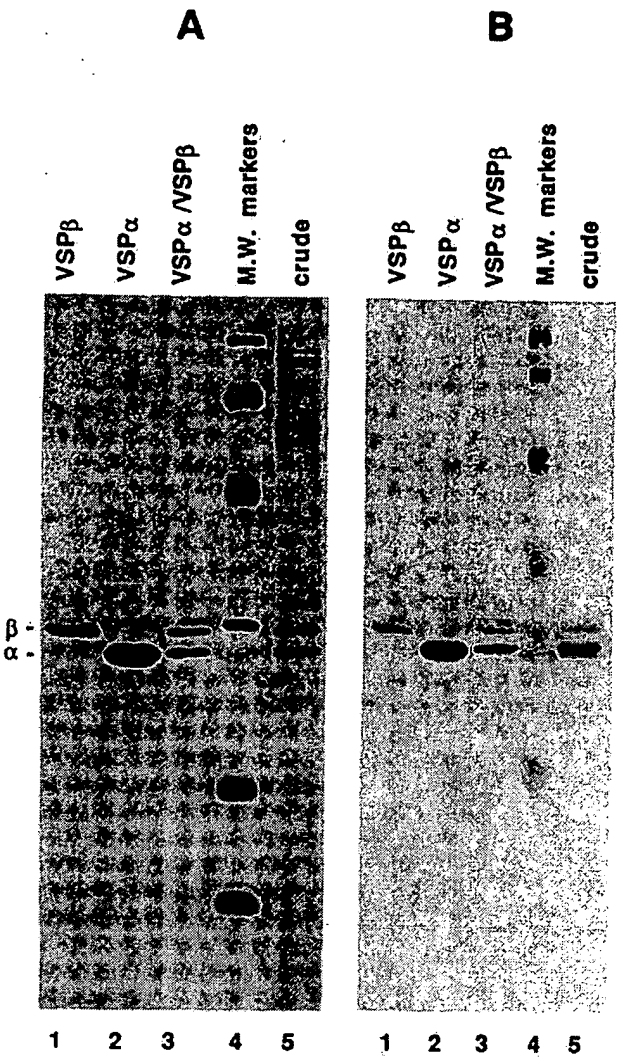

FIG. 2. **Coomassie Blue-stained gel and blotted nitrocellulose filter probed with anti-VSPα and anti-VSPβ antibodies.** Identical samples were electrophoresed on two 12% SDS-PAGE gels. One gel was stained with Coomassie Blue (*A*), and one gel was blotted to nitrocellulose and antibodies to VSPα and VSPβ were incubated with the filter (*B*). Protein bands on the blot were developed using alkaline phosphatase-conjugated secondary antibody. *Lanes 1–3* are purified VSP samples from hypocotyl extracts (VSPα/β and VSPβ) and leaf extracts (VSPα). *Lane 4* shows molecular weight markers (prestained on blot), and *lane 5* contains total soluble protein from hypocotyl tissue.

ent with earlier observations (20, 21).

Anti-VSP Antibodies—In an earlier study, we used antibodies prepared against VSP isolated from soybean (8, 17). As expected, the anti-VSP antibodies were highly reactive against carbohydrate determinants, as well as VSP protein determinants. To circumvent this problem in the present study, we fused portions of the *vspA* and *vspB* open reading frames to *trpE* and overexpressed the fusion proteins in *E. coli*. Antibodies were prepared against the fusion proteins. Mono Q fractions containing VSPα, VSPβ, and VSPα/β dimers and total soluble cell protein were fractionated on SDS-PAGE gels and then stained or blotted for immunodetection with anti-VSP antibodies (Fig. 2). As shown in Fig. 2*B* (*lane 5*), antibodies prepared against the fusion proteins were highly specific against the VSP. The fraction containing VSPβ (Fig. 2*A*, *lane 1*) and VSPα (Fig. 2*A*, *lane 2*) show slight contamination with other proteins. The immunoblot of these same samples verified the identity of the purified proteins and shows that the VSPβ (Fig. 2*B*, *lane 1*) sample contains a very small amount of VSPα.

VSP Exhibit Phosphatase Activity—Mono Q fractions containing VSPα, VSPβ, or VSPα/β were electrophoresed on nondenaturing polyacrylamide gels and silver-stained (Fig. 3*A*) or stained for acid phosphatase activity (Fig. 3*B*). The results of this assay showed that all of the VSP fractions have phosphatase activity. The activity in these fractions co-migrated with silver-stained protein bands (Fig. 3*A*), indicating that the phosphatase activity was due to the VSP. To confirm this, Mono Q-purified VSP was separated on a nondenaturing

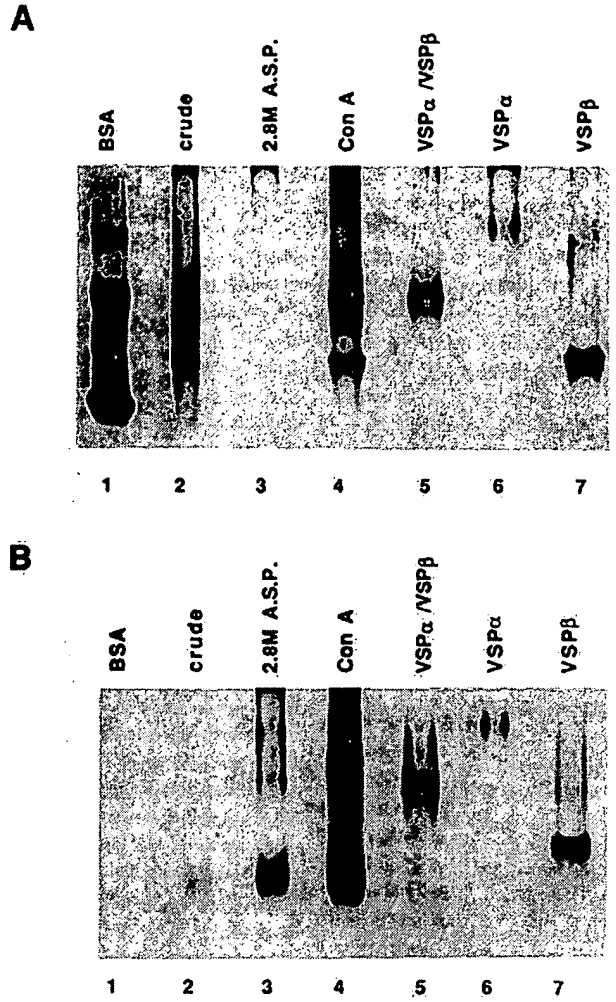

FIG. 3. **Silver stain and acid phosphatase activity stain of VSP-containing samples and bovine serum albumin electrophoresed on 8% nondenaturing gels.** Samples containing VSP (*lanes 2–7*) were electrophoresed on 8% nondenaturing gels and silver-stained (*A*) or stained for acid phosphatase activity (*B*). Gels shown were loaded with the same samples. Bovine serum albumin (*BSA*) was used as a standard (*lane 1*). Samples were loaded on an equal protein basis. Protein in *lane 5* (crude) is from hypocotyl extract before ammonium sulfate fractionation. The lane labeled *2.8M A.S.P.* is the resuspended pellet from the 2.8 M ammonium sulfate precipitation. The lane labeled *ConA* is the eluant of the concanavalin A column. The lanes labeled *VSP* are the purified samples from the Mono Q column.

gel, and unstained regions of the gel that aligned with phosphatase activity-stained bands (VSPα, VSPβ, and VSPα/β) were excised and rerun on a denaturing gel. The resulting proteins were blotted to nitrocellulose and probed with anti-VSP antibodies. This experiment demonstrated that the bands staining on an acid phosphatase activity gel were VSP (data not shown).

VSPα, VSPβ, and VSPα/β exhibited different mobilities when run on nondenaturing gels (Fig. 3). Differences in charge, shape, or aggregation state will influence protein migration in these gels. Spilatro and Anderson (20) and Rapp *et al.* (21) found that the VSP exist as dimers of α_2, β_2, or α/β subunits. Therefore, the differences in migration observed on nondenaturing gels are probably not due to differences in aggregation state. VSPβ dimers, which migrate furthest into the gel, are expected to carry greater negative charge than α/β or α dimers, due to the isoelectric points of the proteins ($\beta = 5.8$, $\alpha = 8.6$). Therefore, charge differences probably explain the differences in migration observed.

VSP Phosphatase Specific Activity and VSPβ Heterogeneity—The specific activities of VSPα, VSPβ, and VSPα/β were determined at their respective pH optima (see below) using *o*-carboxyphenyl phosphate as a substrate. VSPα had low activity relative to VSPβ, and VSPα/β dimers were the most active. The calculated specific activities of VSPα, VSPβ, and VSPα/β were 0.3–0.4 units/mg, 2–4 units/mg, and 7–10 units/mg, respectively, for four different purifications from leaf (α) and hypocotyl (α/β and β).

The differences in activity of individual isolates of VSPβ and VSPα/β may be attributable to the heterogeneity of VSPβ. Mason *et al.* (8) demonstrated that VSPβ can be resolved into at least four apparent isoforms on a two-dimensional polyacrylamide gel. VSPβ heterogeneity was also observed in this study during Mono Q chromatography. Protein from the leading edge of the VSPβ Mono Q peak migrated more slowly in nondenaturing gels than did VSPβ proteins eluting later (Fig. 4). Furthermore, VSPβ proteins with the greatest mobility also exhibited the highest specific activity. VSPα isoelectric point heterogeneity has not been observed (8).

pH Optima—The pH optima of VSP phosphatase activities was determined using *o*-carboxyphenyl phosphate as a substrate (Fig. 5). This analysis showed that VSPα/β and VSPβ exhibit pH optima of approximately 5.0. The pH optimum for VSPα dimers was shifted to approximately pH 5.5, and all three forms of VSP were active over a wide range, retaining greater than 40% activity from pH 4.0 to 6.5.

Inhibitors, Heat Stability, and Metal Dependence—VSPα/β acid phosphatase activity was sensitive to heating (Table I). Activity was also inhibited significantly by NaF and Na_2MoO_4. However, $NaPP_i$ and NaP_i at 5 mM only partially inhibited enzyme activity. At pH 5.0, VSP acid phosphatase activity was unchanged by $MgCl_2$ or $CaCl_2$ and inhibited by $ZnCl_2$. Although the activity of VSPα/β at pH 7.0 is low, no further decrease in activity was observed when 5 mM EDTA was added to the assay buffer. These data suggest that soybean VSP acid phosphatase activity is not metal-dependent. Finally, asparagine, glutamine, and sucrose had no effect on activity under the conditions of the assay.

VSP Substrate Specificity—In order to begin analysis of

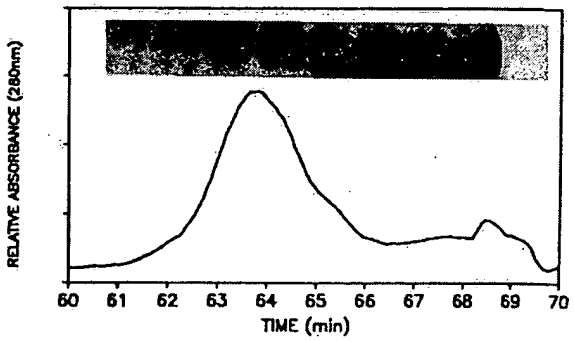

FIG. 4. **Mono Q chromatography elution profile of hypocotyl extract and gel electrophoresis acid phosphatase activity stain of fractions collected.** This figure is an expanded segment of a longer chromatography run (see Fig. 1, *top panel*). *Time* refers to the time after the start of the run. The *inset* is the nondenaturing gel (acid phosphatase activity) analysis of fractions collected during the chromatography run. Samples are from 1-min fractions; therefore, lanes align with times shown on the time scale. The bands with different mobilities on the gel are VSPβ and demonstrate the heterogeneity (apparent multiple isoforms) of VSPβ.

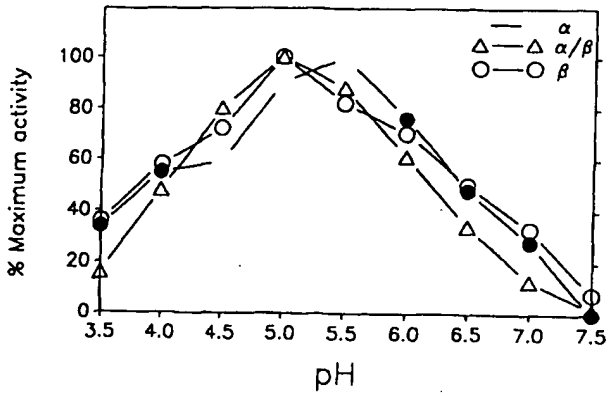

FIG. 5. **Phosphatase activity associated with VSPα, VSPβ, and VSPα/β as a function of pH.** Activity was determined using the conditions described in the text and expressed as a percent of maximum.

TABLE I

Effect of treatments or substances on VSPα/β acid phosphatase activity

Relative activity refers to the percent of the uninhibited VSPα/β phosphatase activity (average 8 units/mg). Assays were performed at 25 °C for 5 or 10 min. Activity was determined by monitoring the release of salicylic acid from *o*-carboxyphenyl phosphate. Data represent the average of three assays.

Treatment or substance	Concentration	Relative activity
	mM	%
Heat treatment (10 min at 70 °C)		0
$NaPP_i$	5	58
NaP_i	5	64
NaF	10	<5
Na_2MoO_4	0.5	<5
$MgCl_2$	5	100
$ZnCl_2$	5	58
$CaCl_2$	5	90
EDTA (pH 7.0)	5	100*
Asparagine	5	100
Glutamine	5	100
Sucrose	10	100

* 100% of the activity at pH 7.0 (activity at pH 7.0 is 13% of activity at pH 5.0).

VSP acid phosphatase function *in vivo*, we analyzed potential substrates for this enzyme (Table II). All substrates were used at a concentration of 5 mM. Of the compounds tested, PEP, ATP, ADP, $NaPP_i$, tripolyphosphate, and tetrapolyphosphate were the most efficiently cleaved substrates. Glucose 6-phosphate, fructose 1,6-bisphosphate, ribose 5-phosphate, and glyceraldehyde 3-phosphate were cleaved less well, and AMP, fructose 6-phosphate and glycerol 2-phosphate were cleaved least efficiently. In general, VSPα, VSPβ, and VSPα/β showed similar relative substrate activities that differed from acid phosphatase-1 in significant ways (Table II). For example, acid phosphatase-1 is highly active on ribose 5-phosphate and glyceraldeyde 3-phosphate (34), whereas the VSP are less active; VSP are highly active on ATP and ADP, whereas acid phosphatase-1 has low activity.

Table III lists the apparent K_m, V_{max}, and specificity constants for the substrates phosphoenolpyruvate, pyrophosphate, and tetrapolyphosphate for purified hypocotyl VSPα/β. VSPα/β phosphatase has the highest specificity constant for tetrapolyphosphate, which indicates that it is the best substrate of the three tested.

DISCUSSION

The results presented in this paper demonstrate that soybean VSPα and VSPβ are acid phosphatases. Purified VSPα, VSPβ, and VSPα/β dimers all exhibit phosphatase activity with pH optima of 5.0–5.5. Of the three forms of VSP dimers, VSPα/β dimers have the highest specific activity, which was 2–3-fold lower than that reported for acid phosphatase-1 (34). The difference in activity between the homodimers and heterodimers of VSP may be significant because different tissues accumulate different ratios of VSPα, VSPβ, and VSPα/β dimers (11). In addition, although only a small portion of the VSP co-fractionate with cell walls, the ratio of VSPα:VSPβ is high in these fractions (8, 17).

The VSP can be distinguished from previously characterized plant acid phosphatases in several ways. First, VSP form dimers of 25–31-kDa subunits (20, 21). This is similar to acid phosphatase-1 but different from soybean cotyledon acid phosphatase (53-kDa nonglycosylated monomer) (37), black mustard acid phosphatase (60-kDa monomer, pI 4.5) (36), PEP-phosphatase (55-kDa monomer) (35), phytase (50-kDa monomer) (38), 3-phosphoglycerate phosphatase (160-kDa tetramer) (39), phosphoglycolate phosphatase (81-kDa dimer) (40), and other acid phosphatases (41–43). VSP acid phosphatase activity does not require metals and is relatively heat-labile. Inhibition of VSP acid phosphatase activity by fluoride and molybdate is common to many acid phosphatases (39). However, VSP phosphatase activity is relatively insensitive to P_i, in contrast to some other acid phosphatases (35, 36), including those induced by P_i starvation (44). Induction of VSP by phosphate starvation has not been examined, although acid phosphatase-1, which is most similar to VSP, is not induced by limiting phosphate (22). Finally, VSP phosphatase, like many other phosphatases, exhibits broad but preferential substrate specificity. Of the substrates tested, VSP phosphatase is most active on ATP, ADP, PP_i, PEP, and tri/tetrapolyphosphates. (Note the V_{max}/K_m data for PEP, pyrophosphate, and tetrapolyphosphate). This substrate specificity is very different from acid phosphatase-1, which only poorly hydrolyzes ATP and ADP. In addition, acid phosphatase-1 has a lower pH optimum of 3.5–4.0 (34).

Speculation on the Function of VSP Phosphatase—The information obtained in this study, combined with previously obtained information on VSP, suggest several possibilities for the role of VSP acid phosphatase activity. The stimulation of *vsp* expression by sucrose, glucose, or fructose and inhibition when plants are grown on limiting nitrogen (13, 14) indicate that if VSP phosphatase activity is important it most likely facilitates the uptake, storage, and assimilation of carbon/nitrogen in apical growing zones. VSP are localized primarily in vacuoles of cells in apical growing regions (leaves, stems, pods, and flowers), which are sinks for carbon and nitrogen (4–6, 11). The *vsp* mRNA accumulate in young leaves and then decline as leaves mature (12). The VSP will reaccumulate during the reproductive phase if flowers or pods are removed or prevented from forming by mutation (1–3, 10). During this latter phase, amino acids and carbon are mobilized and exported from leaves to help support development of reproductive structures. It is also known that the VSP accumulate to the highest levels in leaf PVM and bundle sheath cells (4–6). The PVM and bundle sheath cells are the primary loading and unloading sites for nitrogenous compounds (glutamine, asparagine, and ureides) and sucrose to and from the phloem (4–6). It has been noted that PVM cells are larger than mesophyll cells and have a very large vacuole that probably serves an important storage role in these cells.

The VSP phosphatase hydrolyzes phosphate from sugar-phosphates. However, the VSP phosphatase is different from sucrose phosphate phosphatase, which is inhibited by 10 mM sucrose, and can be distinguished from the PP_i-tonoplast

TABLE II

Comparison of VSP phosphatase activity on different substrates

Data represent efficiency of liberation of P_i (measured spectrophotometrically) from different substrates, as compared to β-napthyl acid phosphate (100%). Substrate concentrations are 5 mM, and digestions were for 60 min. Average activities of enzyme on napthyl acid phosphate were: VSPα/β = 10 units/mg, VSPα = 0.4 units/mg, and VSPβ = 3 units/mg.

Substrate	Relative activity			
	VSPα/VSPβ	VSPα	VSPβ	Acid phosphatase[a]
β-Napthyl acid phosphate	100%	100%	100%	100%
o-Carboxyphenyl phosphate	70			
D-Fructose 6-phosphate	5	15	18	
D-Glucose 6-phosphate	20	13	22	15
Glycerol 2-phosphate	8			
Fructose 1,6-bisphosphate	30			
Ribose 5-phosphate	17			69
Glyceraldehyde 3-phosphate	28		12	73
Dihydroxyacetone phosphate	15			
Phosphoenolpyruvate	90	53	100	
ATP	80	28	60	0
ADP	98	22	75	3
AMP	9	5	9	5
$NaPP_i$	106	19	20	
Tripolyphosphate	110	37	63	
Tetrapolyphosphate	118		68	
Sodium phytate	0			

[a] Values were calculated from Paul and Williamson (34).

TABLE III

Substrate specificity of VSPα/β

The rate of substrate cleavage by 0.01 units of enzyme was measured at substrate concentrations from 1 μM to 10 mM by the following increments: 1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 500 μM, 1 mM, 5 mM, and 10 mM. Assays were performed at pH 5.0, 25 °C, for 5 min.

Substrate	K_m	V_{max}	Specificity constant (V_{max}/K_m)
	mM	*μmol/min/mg*	
Phosphoenolpyruvate	0.420 ± 0.09	4.1 ± 0.2	10 ± 1.8
Pyrophosphate	0.150 ± 0.02	4.2 ± 0.2	28 ± 3.7
Tetrapolyphosphate	0.042 ± 0.008	2.0 ± 0.1	49 ± 8.6

phosphatase (with a broad alkaline pH optimum), which is involved in proton pumping (45). In addition, sugar-phosphates do not readily pass into the vacuole (46). Further, the preference of VSP for PP_i, ADP, ATP, PEP, and polyphosphates suggests that the VSP probably play a different role. Among these substrates, ADP and ATP appear less likely substrates due to their low levels in vacuoles (47).

VSP could be a PEP-phosphatase that in theory could accelerate the synthesis of pyruvate from glucose. The pyruvate produced in this way could be utilized in nitrogen assimilation in PVM cells or to produce other carbon compounds that are transported to mesophyll cells. Hydrolysis of PEP also generates P_i that could be used in the formation of hexose-phosphates from sucrose. A PEP-phosphatase (35) has been isolated and characterized that is different from VSP phosphatase. PEP-phosphatase is a monomer of 55 kDa, whereas VSP form dimers of 25–31-kDa subunits (20, 21). PEP-phosphatase requires metals and is inhibited by 0.5 mM P_i (35). In contrast, VSPα/β phosphatase activity is relatively insensitive to P_i, does not require metal ions, and has a relatively high K_m for PEP (420 *versus* 50 μM, VSP phosphatase *versus* PEP-phosphatase) (35). Moreover, the previously characterized PEP-phosphatase is induced by P_i starvation, apparently to bypass a step in glycolysis that requires adenylates (44). Phosphate starvation results in depressed adenylate pools and the induction of pyrophosphate: fructose 6-phosphate 1-phosphotransferase, nonphosphorylating NADP-glyceraldehyde-3-phosphate dehydrogenase, and PEP-phosphatase. Each of these enzymes bypasses a step in glycolysis requiring adenylates. In contrast, addition of sucrose to soybean cells, a condition required for VSP accumulation, does not depress ATP levels (48). Therefore, if VSP functions as a PEP-phosphatase *in vivo*, it seems likely it functions to supplement the action of pyruvate kinase rather than to bypass this step.

VSP phosphatase showed the highest substrate specificity for tetrapolyphosphate. This was surprising because we were unable to locate information documenting polyphosphates in higher plants. However, polyphosphates are localized in vacuoles of many organisms (49). In *Dunaliella*, hydrolysis of polyphosphates has been implicated in osmotic adjustment in response to salt stress (50). Furthermore, polyphosphate hydrolysis in *Dunaliella* is an important response to amine uptake because it contributes to the maintenance of vacuolar pH needed for ammonium/basic amino acid sequestration in vacuoles (51). As noted earlier, the VSP are localized in vacuoles of PVM cells that play a primary role in amino acid transfer between mesophyll cells and vascular tissue. Therefore, it is reasonable to speculate that the VSP may be polyphosphate exophosphatases that play a role in amino acid uptake and temporary sequestration in PVM cells.

The VSP were so named because they accumulate in PVM vacuoles during periods of high amino acid and carbon flux to and from leaf mesophyll cells (1–6). The finding that VSP has phosphatase activity does not rule out a protein storage function because other vegetative storage proteins, such as patatin, retain enzyme activity (lipid acyl hydrolase) (52). Furthermore, the VSP, especially VSPα, exhibit relatively low activity as compared with other phosphatases. Moreover, VSP phosphatase activity could be regulated through changes in VSPα/VSPβ ratios and as yet undefined modification of VSPβ that results in greater activity in the more negatively charged forms of VSPβ.

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